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(54) Title: COMBINED INHIBITION OF PHOSPHODIESTERASE-4 (PDE-4) AND PHOSPHODIESTERASE-3 (PDE-3) AS A THERAPY FOR TH1-MEDIATED AUTOIMMUNE DISEASES

(57) Abstract: PDE4 inhibitors and PDE3 inhibitors can be administered to modulate immune responses from Th1 toward Th2 phenotype.

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**COMBINED INHIBITION OF PHOSPHODIESTERASE-4 (PDE-4) AND
PHOSPHODIESTERASE-3 (PDE-3) AS A THERAPY FOR Th1-MEDIATED
AUTOIMMUNE DISEASES**

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Background of the Invention

Field of the Invention

The invention relates to the discovery that PDE4 and PDE3 inhibitors used in combination creates a synergistic enhancement of immunomodulatory therapeutic activity.

10 Description of the Related Art

Hyperactive Th1-mediated immune responses are thought to be involved in the pathogenesis of many autoimmune diseases, including multiple sclerosis (MS). Phosphodiesterases (PDE) are enzymes degrading the second messenger cAMP, which mediates and regulates essential intracellular processes (Teixeira, M.M. *et al.* 1997 *Trends Pharmacol Sci* 18:164). There are ten different PDE families, but immune cells predominantly express families PDE4, PDE3 and to a lesser extent PDE7 (Ekholm, D. *et al.* 1997 *J Immunol* 159:1520; Bloom, T.J., and J.A. Beavo 1996 *PNAS USA* 93:14188). Although no PDE 7 inhibitor is available, the inhibitors of PDE4 and PDE3 families exert complex immunomodulatory properties. In animals, PDE4 inhibitors inhibit antigen-mediated T-cell proliferation and skew the T cell cytokine profile toward a Th2-phenotype by downregulating the expression or production of Th1 cytokines (Marcoz, P. *et al.* 1993 *Mol Pharmacol* 44:1027; Sommer, N. *et al.* 1997 *J Neuroimmunol* 79:54; Eigler, A. *et al.* 1998 *J Leukoc Biol* 63:101) and have no effect or even augment the production of Th2 cytokines (Lacour, M. *et al.* 1994 *Int Immunol* 6:1333; Eigler, A. *et al.* 1998 *J Leukoc Biol* 63:101). These properties render PDE-inhibition a candidate therapy for Th1-mediated autoimmune disorders. Indeed, both non-selective as well as PDE4-specific inhibitors were effective in ameliorating disease in different experimental autoimmune encephalomyelitis (EAE) models (Rott, O. *et al.* 1993 *Eur J Immunol* 23:1745; Sommer, N. *et al.* 1995 *Nat Med* 1:244; Genain, C.P. *et al.* 1995 *PNAS USA* 92:3601; Sommer, N. *et al.* 1997 *J Neuroimmunol* 79:54) and in collagen-induced arthritis models (Ross, S. E. *et al.* 1997 *J Immunol* 159:6253; Nyman, U. *et al.* 1997 *Clin Exp Immunol* 108:415). However, the simple extrapolation of therapeutic efficacy from animal models to human disorders is not

easily feasible (Panitch, H.S. *et al.* 1987 *Lancet* 1:893), and therefore the analysis of the immunomodulatory properties of PDE inhibitors on human immune cells is an important step in pre-clinical testing. Studies exploring the effect of PDE inhibitors in humans *in vitro* or *in vivo* are still limited. While there is some evidence for a preferential inhibition of proinflammatory cytokines in Th1-mediated human autoimmune conditions (Rieckmann, P. *et al.* 1996 *J Neuroimmunol* 64:193; Ekholm, D. *et al.* 1997 *J Immunol* 159:1520; Pette, M. *et al.* 1999 *J Neuroimmunol* 98:147), data from asthmatic and atopic individuals reached almost the opposite conclusion, i.e., that these drugs lead to preferential inhibition of Th2 responses (Essayan, D.M. *et al.* 1997 *J Pharmacol Exp Ther* 282:505).

Summary of the Invention

Phosphodiesterase (PDE) 4 inhibitors have the potential to modulate immune responses from Th1 toward Th2 phenotype, especially in individuals with dysregulated immune system, and are therefore considered candidate therapies for Th1-mediated autoimmune disorders. However, depending on the model and cell types employed, studies of atopic individuals have come to the opposite conclusion, i.e. that PDE inhibitors may modulate immune responses from Th2 to Th1 phenotype and therefore may be beneficial in asthma. Using *in vitro* immunopharmacological techniques we analyzed the effects of PDE4 and PDE3 inhibitors on human immune cells in order to address these discrepancies and broaden our understanding of their mechanism of action. Our results indicate that PDE inhibitors have complex inhibitory effects within *in vivo* achievable concentration ranges on Th1-mediated immunity whereas Th2-mediated responses are mostly unaffected or enhanced. The Th2-skewing of the developing immune response is explained by effects of PDE inhibitors on several factors contributing to T cell priming: the cytokine milieu, the type of costimulatory signal, i.e., upregulation of CD86, downregulation of CD80, and the antigen avidity. The combination of PDE4 and PDE3 inhibitors expresses synergistic effects and may broaden the therapeutic window. Finally, we observed a differential sensitivity to PDE-inhibition in autoreactive versus foreign-antigen-specific T cells and cells derived from MS patients versus those derived from healthy donors. This suggests that PDE inhibition weakens the strength of the T cell stimulus and corrects the underlying disease-associated cytokine skew in T cell-mediated autoimmune disorders. These new findings broaden the understanding of the immunomodulatory actions of PDE inhibitors and underscore their promising drug profile for the treatment of autoimmune disorders.

Brief Description of the Drawings**Figure A: Selection of Th1 or Th2 mediated immunity**

In T helper (Th) cell mediated immunity, either the Th1 or Th2 pathway dominates immune response. Whether the "naive" T cell differentiates into Th1 or Th2 phenotype depends on complex "priming conditions", which include cytokine milieu, costimulatory signal and strength and quality of T cell receptor stimulus. Regulation of Th cells is in part maintained by cytokines specific to each of the Th1 and Th2 pathways. IFN- γ is the main cytokine product of the Th1 pathway, while IL-4 is the main cytokine product of the Th2 pathway. IFN- γ and IL-4 regulate Th differentiation through inhibitory action. IFN- γ inhibits the Th2 mediated pathway and IL-4 inhibits the Th1 mediated pathway. Using this system, healthy immune systems are able to choose the pathway most appropriate to combat different types of antigens. Patients afflicted with Th1 mediated autoimmune diseases have overactive Th1 cells which dominate, using inhibitory action, over the Th2 pathway (Roitt, et al. 1993 *Immunology*, 3rd Edition 8.1-8.15).

Figure B: Affect of PDE4 inhibitors (Rolipram) on Th1 and Th2 mediated immunity

PDE4 inhibitors (e.g., rolipram) have an inhibitory affect on the production of IFN- γ , but not IL-4. By inhibiting the production of IFN- γ , the Th1 pathway is downmodulated causing, e.g., decreased macrophage function, decreased production of Th1 cytokines and diminished cytotoxicity. Meanwhile, normal IL-4 production further inhibits the Th1 pathway. Through this mechanism PDE4 inhibitors modulate immune responses from the Th1 toward the Th2 phenotype. In laboratory studies, *Rolipram* inhibited IFN- γ production by 12.26 - 27.8 % leading to a 8.8 - 32 % inhibition in the proliferation of antigen-specific T Cell Lines.

Figure C: Affect of PDE3 inhibitors (cilostamide) on Th1 and Th2 mediated immunity

PDE3 inhibitors (e.g., cilostamide) have no significant inhibitory affect on the production of IFN- γ , but in higher doses enhance IL-4 production. Higher IL-4 production inhibits the Th1 pathway. Through this mechanism PDE3 inhibitors modulate immune responses from the Th1 toward the Th2 phenotype. In laboratory studies, cilostamide enhances IL-4 production up to 11.39% while inhibiting T cell proliferation by 6.31 - 22.61%.

Figure D: Affect of the combination of PDE4 (rolipram) and PDE3 (cilostamide) on Th1 and Th2 mediated immunity

Together, PDE4 inhibitors (rolipram) and PDE3 inhibitors (cilostamide), have significant inhibitory effect on the production of IFN- γ , and no effect or even a slight enhancement effect on IL-4. By inhibiting the production of IFN- γ , the Th1 pathway is disrupted causing, e.g., decreased function of macrophages, decreased production of Th1 cytokines and diminished cytotoxicity. Meanwhile, normal production or even slightly enhanced production of IL-4 further inhibits the Th1 pathway. Through this mechanism PDE4 and PDE3 inhibitors modulate immune responses from the Th1 toward the Th2 phenotype. In combination PDE4 and PDE3 inhibitors have an inhibitory effect on IFN- γ unexpectedly greater than the predicted additive inhibitory effect of PDE4 inhibitors and PDE3 inhibitors individually. The degree of synergism between PDE4 inhibitor (rolipram) and PDE3 inhibitor (cilostamide) based on Berenbaum equation calculated from our proliferation data was 8.9. Values below 1 indicate antagonism, 1 indicates additivity, and values higher than 1 indicate synergism. The higher the numeric value, the higher is the degree of synergism between two drugs. In laboratory studies the aforementioned synergistic effect caused by the combination of PDE4 inhibitors (rolipram) and PDE3 inhibitors (cilostamide) results in the inhibition of IFN- γ production by 16.07 - 46.3 % leading to a 10.34 - 61.05 % inhibition in the proliferation of antigen-specific T Cell Lines. Inhibition in IL-4 production ranged from -0.62 - 1.69 %.

Figure 1: Effect of PDE inhibitors on antigen-specific proliferation of TCL

A total of 47 TCL was stimulated with specific antigen without or with three increasing concentrations of rolipram and cilostamide. For the combination of rolipram and cilostamide, each drug was used in concentration 0.05, 0.5 and 5 μ M respectively. Results are depicted as counts per minute (CPM). Statistically significant differences are marked by star (* $P < 0.05$, ** $P < 0.01$). Only rolipram and the combination of both drugs had a dose-related inhibitory effect on antigen-specific TCL proliferation. The combination of rolipram and cilostamide was most potent, demonstrating a synergistic effect of individual drugs. The differences between three treatment modalities reached statistical significance ($P < 0.05$; Repeated measures ANOVA analysis).

Figure 2: Effect of PDE inhibitors on proliferation of TCL stratified based on their origin (derived from MS patients versus healthy donors) and selecting antigen (autoreactive versus foreign-antigen specific)

Upon stratification of the proliferation data with respect to the origin of the TCL and the type of the selecting antigen, a hierarchy of the sensitivity to PDE-inhibition emerged; MS-derived-TCL were more sensitive than healthy donor (HD)-derived TCL. A similar differential sensitivity to PDE4 versus PDE3 inhibition was observed in the analysis of autoreactive and foreign-antigen-specific TCL within each population group. Statistically significant differences are marked with a star ($P < 0.05$; Friedman's repeated measures analysis on ranks).

Figure 3: Effect of PDE inhibitors on cytokine production of antigen-specific TCL

The effect of rolipram, cilostamide and their combination on antigen-specific production of IFN- γ and IL-4 was assessed in parallel to the proliferation. Results are depicted as percentage of inhibition of cytokine-production for each concentration of drug as compared to control cultures (no drug added). Statistically significant differences are marked with stars (* $P < 0.05$, ** $P < 0.01$). While all TCL tested in proliferation assays produced IFN- γ and are included in the analysis, only 9/26 MS-derived TCL and 17/21 healthy donor-derived TCL produced IL-4 and therefore only these 26 TCL could be included in the analysis of the effect of PDE-inhibitors on IL-4 production. Only rolipram and the combination of rolipram with cilostamide inhibited the production of IFN- γ significantly as compared to control samples. This inhibition paralleled the effect on TCL proliferation and was clearly strongest for the combination of both drugs. Neither rolipram nor the drug combination has a significant effect on IL-4 production. High concentration (10 μ M) of cilostamide enhanced IL-4 production, despite the demonstrated inhibitory effect on proliferation. Differences between individual drugs were statistically significant ($P < 0.05$).

Figure 4: Effect of PDE inhibitors on functional antigen-avidity

A total of 15 TCL was tested in antigen dose-response assays in the presence of 1 μ M concentration of drug and a wide antigen-concentration range. Only three TCL are depicted in this figure. Th1:MBP (TCL #9 in Table 1) and Th0:Flu-HA (TCL #5 in Table 1) were selected as a representative TCL for 14 MBP- or Flu-HA-specific TCL tested. The Th2:Cop-1 (TCL #15 in Table 1) TCL was added for this assay only, as none of the 47 MBP- or Flu-HA-specific TCL tested in previous experiments expressed a clear Th2 phenotype. The EC₅₀ (antigen concentration leading to 50% maximal proliferation of TCL) were calculated from these dose-response curves for each TCL and are summarized in

Table 1. The TCL depicted in this figure demonstrate the hierarchy of potency of the immunomodulatory effect between individual drugs. The combination of rolipram and cilostamide had the strongest inhibitory effect on T-cell proliferation and IFN- γ secretion, while having no effect or enhancing the production of IL-4. The differential susceptibility to PDE inhibitor-mediated immunomodulation on TCL was noted, based on their cytokine-phenotype, with Th1-TCL being more susceptible than Th0 or Th2 -TCL. Overall, the magnitude of the effects of PDE inhibitors on individual TCL was similar through the tested antigen concentration range.

Figure 5: Effect of rolipram on the expression of costimulatory molecules by antigen-presenting cells

The surface expression of MHC class II and costimulatory molecules CD80 and CD86 on monocytes, B- and T-lymphocytes was assessed by triple-staining flow-cytometry analysis. Resting or non-specifically activated (LPS or PHA) PBMC were incubated for 12 hours in bulk culture with or without rolipram and analyzed by flow cytometry. Only the effect on monocytes (gated based on size-characteristics and CD14 expression), both rested and activated by LPS, is depicted in this figure, but similar results were obtained upon activation by PHA or when gating of B- or T-lymphocytes. Rolipram downregulates MHC class-II expression both on resting and activated cell, while it downregulates CD80 and upregulates CD86 expression upon activation with either non-specific stimulus. These changes were observed both in MS patients and healthy donors.

Figure 6: Immunomodulatory effect of PDE inhibitors within in vivo achievable concentration ranges

The concentration of rolipram that can be achieved in humans in steady-state without major side-effects ranges from 0.09 to 0.2 μ M. At concentration of 0.1 μ M, PDE inhibitors express mild, but multi-level immunomodulatory profile by suppressing antigen-specific proliferation and IFN- γ production preferentially of autoreactive TCL, while not affecting, or even enhancing IL-4 production. This immunomodulation is strongest for the combination of rolipram and cilostamide demonstrating the synergistic effect. Statistically significant effect as compared to control cultures is marked by stars (* P < 0.05, ** P < 0.01).

Table 1: Effect of PDE inhibitors on TCL antigen-avidity (EC₅₀):

TCL origin	Antigen	TCL	Phenotype	EC ₅₀ (μg/ml)			
				No drug	Cilostamide	Rolipram	Rolipram +
				Cilostamide			
1	Flu-HA	MS	Th1	0.070	0.070	0.080	0.200
2	Flu-HA	MS	Th1	0.070	0.200	0.200	0.200
3	Flu-HA	MS	Th1	0.009	0.010	0.030	0.500
4	Flu-HA	MS	Th0	0.003	0.003	0.100	0.100
5	Flu-HA	MS	Th0	0.008	0.010	0.100	0.100
6	MBP	MS	Th1	0.050	0.070	0.070	0.100
7	MBP	MS	Th1	10.000	10.000	100% inhibition	100% inhibition
8	MBP	MS	Th1	2.000	3.000	2.000	3.000
9	MBP	MS	Th1	1.500	1.500	1.500	1.400
10	MBP	HD	Th1	20.000	20.000	20.000	30.000
11	MBP	MS	Th0	3.000	3.000	10.000	100% inhibition
12	MBP	MS	Th0	1.000	1.500	1.000	1.000
13	MBP	HD	Th0	25.000	25.000	25.000	25.000
14	MBP	HD	Th0	3.000	3.000	4.000	3.000
15	Cop-1	HD	Th2	3.000	3.000	3.000	3.000
Total		Mean+/-SD		4.58+/-7.77	4.69+/-7.73 *	4.79+/-8.02 *	5.20+/-10.02 *

* P<0.05; Friedman's repeated measures analysis on ranks with Student-Newman-Keuls test

Detailed Description of the Preferred Embodiment

It has been recognized that the immunomodulatory profile of PDE4 inhibitors makes them a potential therapy for human Th-1 mediated autoimmune diseases and such therapies are currently in a stage of pre-clinical development. We have extensively studied the immunomodulatory characteristics of a prototypic PDE4 and PDE3 inhibitors and their combination on human autoreactive lymphocytes derived from MS patients and healthy donors and provided experimental evidence that the combination of PDE4 and PDE3 inhibition is more potent and more favorable with respect to the immunomodulatory potential for treatment of Th-1 mediated autoimmune disease than the PDE4 inhibition alone. It is questionable, whether adequate PDE4 inhibitor levels, which would exert immunomodulatory functions can be achieved in humans due to side effect profile (mainly nausea and vomiting). Therefore it is envisioned that the synergistic activity of the two types of inhibitors will allow using significantly lower doses of each type of inhibitor, even if PDE3 inhibitor is used in sub-therapeutic doses in these combinations, and that the immunomodulation will be much stronger, while side effect profile will be more favorable. The addition of PDE3 inhibitory activity (even in less than 25% of the total PDE-inhibitory activity of the mixture) significantly enhances the efficacy of immunomodulation and thus represents a means for broadening the therapeutic window.

Type 4 phosphodiesterase inhibitors

By "Type 4 phosphodiesterase inhibitor", "specific Type 4 phosphodiesterase inhibitor", "PDE4 inhibitor", and similar expressions are meant a selective, i.e., specific, such inhibitor, where the compound binds to or inhibits preferentially the Type 4 phosphodiesterase when compared to known types of phosphodiesterase types, e.g., 1, 2, 3, or 5 e.g., whereby the compound has a lower IC_{50} (more potent) for the Type 4 phosphodiesterase, such as where the IC_{50} is, e.g., 2-fold, 5-fold, 10-fold, 50-fold, or more potent, for the Type 4 phosphodiesterase compared to another known type of phosphodiesterase, e.g., 1, 2, 3, or 5. Such selectivity of a compound according to the present invention for a Type 4-phosphodiesterase can also be conferred by other means, such as the manner in which it is delivered to its target, e.g., the compound can be associated with an agent which targets it to a specific tissue or cell type having the Type 4 phosphodiesterase; the manner in which it interacts with the host's metabolism and/or physiology; or synthesizing PDE inhibitor prodrugs where activation of the PDE inhibitor is

accomplished by enzymes present in the desired cells or tissues but absent in others. Other compounds and compositions are known and will be readily apparent to those skilled in the art, once armed with the present disclosure. PDE's are enzymes degrading the second messenger cAMP, which mediates and regulates essential intracellular processes.

5 PDE4 inhibitors useful in the methods, compositions and pharmaceutical kits of the present invention include, but are not limited to rolipram, which is comprised of (+) and (-) racemates of 4-[(3-cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone, compounds described in WO93/19068, compounds RO 20-1724 (4-[(3-butyloxy-4-methoxyphenyl)methyl]-2-imidazolidinone), ICI 63197 (2-amino-6-methyl-4-propyl[1,2,4]triazolo[1,5-a]pyrimindin-5(4H)-one), denbufylline, EMD 54622, denbufylline, etazolate, Org 30029, and zardaverine, according to Nicholson et al., *Trends Pharmacol. Sci.*, 12:19-27 (1991). These, and other PDE4 inhibitors will be readily apparent to those skilled in the art, once armed with the present disclosure.

PDE4 inhibitors as treatment in MS and other autoimmune diseases

15 According to the present invention, a pharmaceutical composition comprising an effective amount of a PDE4 inhibitor in combination with a PDE3 inhibitor can be administered to patients having multiple sclerosis, e.g., multiple sclerosis variants such as Neuromyelitis Optica (Devic's Disease), Diffuse Sclerosis, Transitional Sclerosis, Acute Disseminated Encephalomyelitis, and Optic Neuritis, as well as other Th1 mediated diseases including, but not limited to, Diabetes Mellitus, Rheumatoid Arthritis, Uveitis, Inflammatory polyneuropathies and even to other diseases with dysregulated immune system like inflammatory colitis, Systemic lupus erythematosus, Sydenham chorea and PANDAS (Pediatric autoimmune neuropsychiatric disorders associated with Streptococcal infections), Paraneoplastic immune-mediated diseases, Neuroborreliosis, Immune-mediated vasculitides, Sjorgen's syndrome, Behcet's disease and Asthma.

Symptoms ameliorated by PDE4 inhibitors

20 Symptoms of MS, and other Th1 mediated diseases, which are prevented or ameliorated or treated include: weakness and/or numbness in one or more limbs; tingling of the extremities and tight band-like sensations around the trunk or limbs; dragging or poor control of one or both legs to spastic or ataxic paraparesis; hyperactive tendon reflexes; disappearance of abdominal reflexes; Lhermitte's sign; retrobulbar or optic neuritis; unsteadiness in walking; brain stem symptoms (diplopia, vertigo, vomiting); disorders of

5 micturition; hemiplegia; trigeminal neuralgia; other pain syndromes; nystagmus and ataxia; cerebellar-type ataxia; Charcot's triad; diplopia; bilateral internuclear ophthalmoplegia; myokymia or paralysis of facial muscles; deafness; tinnitus; unformed auditory hallucinations (because of involvement cochlear connections); vertigo and vomiting (vestibular connections); transient facial anesthesia or of trigeminal neuralgia; bladder dysfunction; euphoria; depression; dementia, dull, aching pain in the low back; sharp, burning, poorly localized pains in a limb or both legs and girdle pains; abrupt attacks of neurologic deficit; dysarthria and ataxia; paroxysmal pain and dysesthesia in a limb; flashing lights; paroxysmal itching; fatigue; Sjogren's syndrome; scaling; frequent 10 urination; increased thirst; sudden confusion; diarrhea; nausea; abdominal cramps; and/or tonic seizures, taking the form of flexion (dystonic) spasm of the hand, wrist, and elbow with extension of the lower limb. A patient having MS, or other Th1 mediated disease, may have one or more of these symptoms or other clinical manifestations typically 15 associated with MS, or other Th1 mediated disease, and one or more can be ameliorated by administration of compounds according to the present invention.

20 The administration of Type 4 phosphodiesterase inhibitors, such as rolipram, in combination with Type 3 phosphodiesterase inhibitors, can also block or reduce the physiological and pathogenic deterioration associated with MS, e.g., inflammatory response in the brain and other regions of the nervous system, breakdown or disruption of the blood-brain barrier, appearance of lesions in the brain, tissue destruction, demyelination, 25 autoimmune inflammatory response, acute or chronic inflammatory response, neuronal death, and/or neuroglia death.

25 Effects of the administration of rolipram and other Type 4 phosphodiesterase inhibitors, in combination with Type 3 phosphodiesterase inhibitors, include, e.g., preventing the disease, ameliorating symptoms of the disease, reducing the annual exacerbation rate (i.e., reducing the number of episodes per year), slowing the progression 30 of the disease, or reducing the appearance of brain lesions (e.g., as identified by MRI scan). The episodic recurrence of the mentioned diseases such as MS can be ameliorated, e.g., by decreasing the severity of the symptoms (such as the symptoms described above) associated with the, e.g., MS episode, or by lengthening the time period between the occurrence of episodes, e.g., by days, weeks, months, or years, where the episodes can be characterized by the flare-up and exacerbation of disease symptoms, or preventing or slowing the appearance

of brain inflammatory lesions. See, e.g., Adams, R.D., *Principles of Neurology*, 1993, page 777, for a description of a neurological inflammatory lesion.

PDE4 inhibitors activity

The specific inhibition of a Type 4 phosphodiesterase can be measured conventionally, e.g., according to the methods described in Reeves *et al.* 1977 *Biochem J* 241:535-541; by macrophage assay, as described, e.g., in Schade *et al.* 1993 *Eur J Pharmacol* 230:9-14; or WO 93/19068. For a review of phosphodiesterase specificity and how to determine it, see, e.g., Nicholson *et al.* 1991 *Trends Pharmacol Sci* 12:19-27.

The activity of this invention of Type 4 phosphodiesterase inhibitors such as rolipram can be detected, for example, in animals suffering from Experimental Allergic Encephalomyelitis (EAE), an experimental T-lymphocyte initiated disease of the CNS. It can be produced, e.g., in rodents, guinea pigs, rabbits, and primates, by, e.g., immunizing animals with myelin, e.g., from a human brain, and/or corticosteroid administration over a long period of time. It can also be produced by injecting an animal with T-lymphocytes obtained from an animal suffering from EAE.

In particular, the activity can be detected in *Callithrix jacchus* (common marmoset) which has been immunized with myelin, e.g., from a human brain. The *Callithrix jacchus* develops EAE with essentially similar histopathology and neurological symptoms as those at certain stages of the human disease, MS.

Type 3 phosphodiesterase inhibitors

By "Type 3 phosphodiesterase inhibitor", "specific Type 3 phosphodiesterase inhibitor", "PDE3 inhibitor", and similar expressions are meant a selective, i.e., specific, such inhibitor, where the compound binds to or inhibits preferentially the Type 3 phosphodiesterase when compared to known types of phosphodiesterase types, e.g., 1, 2, 4, or 5, e.g., whereby the compound has a lower IC₅₀ (more potent) for the Type 3 phosphodiesterase, such as where the IC₅₀ is, e.g., 2-fold, 5-fold, 10-fold, 50-fold, or more potent, for the Type 3 phosphodiesterase compared to another known type of phosphodiesterase, e.g., 1, 2, 4, or 5. Such selectivity of a compound according to the present invention for a Type 3 phosphodiesterase can also be conferred by other means, such as the manner in which it is delivered to its target, e.g., the compound can be associated with an agent which targets it to a specific tissue or cell type having the Type 3 phosphodiesterase; the manner in which it interacts with the host's metabolism and/or

physiology; or synthesizing PDE inhibitor prodrugs where activation of the PDE inhibitor is accomplished by enzymes present in the desired cells or tissues but absent in others. Other compounds and compositions are known and will be readily apparent to those skilled in the art, once armed with the present disclosure. PDE's are enzymes degrading the second messenger cAMP and cGMP, which mediate and regulate essential intracellular processes.

5 PDE3 inhibitors useful in the methods, compositions and pharmaceutical kits of the present invention include, but are not limited to cilostamide, milrinone, piroximone, pimobendan, imazodan, zardaverine, enoximone, indolidan, motapizone, SK&F94120, RS82856, ORg 30029, ICI 118233, and EMD 54622 according to Nicholson *et al.* 1991
10 *Trends Pharmacol Sci* 12:19-27. These and other PDE3 inhibitors will be readily apparent to those skilled in the art, once armed with the present disclosure.

PDE3 inhibitors as treatment in MS and other autoimmune diseases

According to the present invention, a pharmaceutical composition comprising an effective or even sub-therapeutic amount of a PDE3 inhibitor in combination with an effective dose of PDE4 inhibitor can be administered to patients having multiple sclerosis, e.g., multiple sclerosis variants such as Neuromyelitis Optica (Devic's Disease), Diffuse Sclerosis, Transitional Sclerosis, Acute Disseminated Encephalomyelitis, and Optic Neuritis, as well as other Th1 mediated diseases including, but not limited to, Diabetes Mellitus, Rheumatoid Arthritis, Uveitis, Inflammatory polyneuropathies and even to other diseases with dysregulated immune system like inflammatory colitis, Systemic lupus erythematosus, Sydenham chorea and PANDAS (Pediatric autoimmune neuropsychiatric disorders associated with Streptococcal infections), Paraneoplastic immune-mediated diseases, Neuroborreliosis, Immune-mediated vasculitides, Sjorgen's syndrome, Behcet's disease and Asthma..

25 Symptoms ameliorated by PDE3 inhibitors

Symptoms of MS, and other Th1 mediated diseases, which are prevented or ameliorated or treated include: weakness and/or numbness in one or more limbs; tingling of the extremities and tight band-like sensations around the trunk or limbs; dragging or poor control of one or both legs to spastic or ataxic paraparesis; hyperactive tendon reflexes; disappearance of abdominal reflexes; Lhermitte's sign; retrobulbar or optic neuritis; unsteadiness in walking; brain stem symptoms (diplopia, vertigo, vomiting); disorders of micturition; hemiplegia; trigeminal neuralgia; other pain syndromes; nystagmus and ataxia;

5 cerebellar-type ataxia; Charcot's triad; diplopia; bilateral internuclear ophthalmoplegia; myokymia or paralysis of facial muscles; deafness; tinnitus; unformed auditory hallucinations (because of involvement cochlear connections); vertigo and vomiting (vestibular connections); transient facial anesthesia or of trigeminal neuralgia; bladder dysfunction; euphoria; depression; dementia, dull, aching pain in the low back; sharp, burning, poorly localized pains in a limb or both legs and girdle pains; abrupt attacks of neurologic deficit; dysarthria and ataxia; paroxysmal pain and dysesthesia in a limb; flashing lights; paroxysmal itching; fatigue; Sjogren's syndrome; scaling; frequent urination; increased thirst; sudden confusion; diarrhea; nausea; abdominal cramps; and/or 10 tonic seizures, taking the form of flexion (dystonic) spasm of the hand, wrist, and elbow with extension of the lower limb. A patient having MS, or other Th1 mediated disease, may have one or more of these symptoms or other clinical manifestations typically associated with MS, or other Th1 mediated disease, and one or more can be ameliorated by administration of compounds according to the present invention.

15 The administration of Type 3 phosphodiesterase inhibitors such as cilostamide, in combination with Type 4 phosphodiesterase inhibitors, can also block or reduce the physiological and pathogenic deterioration associated with MS, e.g., inflammatory response in the brain and other regions of the nervous system, breakdown or disruption of the blood-brain barrier, appearance of lesions in the brain, tissue destruction, demyelination, 20 autoimmune inflammatory response, acute or chronic inflammatory response, neuronal death, and/or neuroglia death.

25 Effects of the administration of cilostamide and other Type 3 phosphodiesterase inhibitors, in combination with Type 4 phosphodiesterase inhibitors, include, e.g., preventing the disease, ameliorating symptoms of the disease, reducing the annual exacerbation rate (i.e., reducing the number of episodes per year), slowing the progression of the disease, or reducing the appearance of brain lesions (e.g., as identified by MRI scan). The episodic recurrence of the mentioned diseases such as MS can be ameliorated, e.g., by 30 decreasing the severity of the symptoms (such as the symptoms described above) associated with the, e.g., MS episode, or by lengthening the time period between the occurrence of episodes, e.g., by days, weeks, months, or years, where the episodes can be characterized by the flare-up and exacerbation of disease symptoms, or preventing or slowing the appearance

of brain inflammatory lesions. See, e.g., Adams, R. D. 1993 *Principles of Neurology*, page 777, for a description of a neurological inflammatory lesion.

PDE3 inhibitors activity

The specific inhibition of a Type 3 phosphodiesterase can be measured conventionally, e.g., according to the methods described in Reeves *et al.* 1977 *Biochem J* 241:535-541; by macrophage assay, as described, e.g., in Schade *et al.* 1993 *Eur J Pharmacol* 230:9-14; or WO 93/19068. For a review of phosphodiesterase specificity and how to determine it, see, e.g., Nicholson *et al.* 1991 *Trends Pharmacol Sci* 12:19-27.

Method of synergistic combination therapy

The present invention provides a method of treating MS and other Th1 mediated autoimmune diseases in a mammal comprising administering to the mammal, in combination, a therapeutically effective amount of: (i) at least one PDE4 inhibitor; and (ii) at least one PDE3 inhibitor.

In the present invention, the applicant further claims that the administration of a PDE4 inhibitor (component (i)) in combination with a PDE3 inhibitor (component (ii)) does result in an unexpected synergistic effect in the treatment of Th1 mediated autoimmune diseases. Thus, the result is an unexpected synergistic effect in the modulation of immune responses from Th1 toward Th2 phenotype when a PDE4 inhibitor is administered in combination with a PDE3 inhibitor is greater than the additive effect of each agent when administered alone. This is a remarkable and unexpected effect in view of what is currently known in the literature.

Thus, the present invention claims that a PDE4 inhibitor may be administered in combination with a PDE3 inhibitor, thereby reducing the doses of each drug required to achieve modulation from the Th1 toward the Th2 immune response. Moreover, the present invention claims that the use of the compounds of component (i) and component (ii) of the invention in combination results in a greater than additive effect. Thus, the combination treatment of the present invention of components (i), and (ii) permits the use of lower doses of each component, with reduced adverse, toxic effects of each component and enhanced efficacy of immunomodulation. It thus provides for a greater window of efficacy, since the same maximum tolerated doses can be administered before toxic effects associated with each agent are observed. A lower dosage minimizes the potential of side effects of the

compounds, thereby providing an increased margin of safety relative to the margin of safety for each component when used as a single agent.

In the method of the present invention, the PDE4 inhibitor (such as rolipram) may be administered in combination with a PDE3 inhibitor (such as cilostamide) to achieve a synergistic modulation of Th1 mediated immunity. Synergy occurs when the effect of the compounds, when administered in combination, is greater than the additive effect of the compounds when administered alone as a single agent. In general, a synergistic effect is most clearly demonstrated at suboptimal concentrations of the compounds.

The method of the present invention provides for an enhanced effect of the two drugs when administered in combination. Thus, the claimed combination treatment allows for the use of lowered clinical doses and increases the window of efficacy. In view of the marginal effects associated with the presently approved therapies for treating MS and other Th1 mediated autoimmune diseases, the present invention provides an important advantage over current therapies.

15 Dosage and Formulation

By "therapeutically effective amount" it is meant an amount of component (i), and component (ii) that when administered alone or in combination to a mammal is effective to treat Th1 mediated autoimmune diseases, such as by modulating immune responses from Th1 toward Th2 phenotype. Compositions of the invention present the opportunity of obtaining significant therapeutic benefits in patients with Th1 mediated autoimmune diseases with reduced dosages of PDE4 inhibitor and PDE3 inhibitor, thereby diminishing the side effects and possible toxicity which would result from the otherwise required amounts of the individual drug components.

By "administered in combination", or the like, when referring to component (i), and component (ii), of the present invention, it is meant that the components are administered concurrently to a mammal being treated. By concurrently, it is meant that each component may be administered at the same time or sequentially in any order at different points in time. However, if not administered at the same time, they should be administered sufficiently closely in time so as to provide the desired treatment effect. Suitable dosing intervals and dosing order with such compounds will be readily apparent to those skilled in the art, once armed with the present disclosure. Preferably, all components are

administered at the same time, and if not administered at the same time, preferably they are all administered less than one hour apart from one another.

The present invention also includes pharmaceutical compositions (that is, combination products), such pharmaceutical compositions (combination products) comprising, a PDE4 inhibitor (such as rolipram), and a PDE3 inhibitor (such as cilostamide). Such compositions may be in solid, liquid, sustained release such as transdermal, transnasal, or depot dosage units and may further include a suitable pharmaceutical carrier.

Component (i) of the present invention may also be provided as a pharmaceutical composition comprising a therapeutically effective amount of a PDE4 inhibitor and a pharmaceutically acceptable carrier. Component (ii) of the present invention may likewise be presented as a pharmaceutical composition comprising a therapeutically effective amount of PDE3 inhibitor and a pharmaceutically acceptable carrier. Mixtures of the components (i), and (ii) with or without a pharmaceutically acceptable carrier, are also within the ambit of the present invention.

As will be appreciated by a medical practitioner skilled in the art, the dosage of the combination therapy of the invention may vary depending upon various factors such as the pharmacodynamic characteristics of the particular agent and its mode and route of administration, the age, health and weight of the recipient, the nature and extent of the symptoms, the kind of concurrent treatment, the frequency of treatment, and the effect desired, as described above.

The dosage of the pharmaceutical composition can vary according to, e.g., the manner of administration, the disease being treated and its severity, the overall health and condition of the patient, the age of the patient or other usual criteria. Total dosages of phosphodiesterase inhibitors for all uses mentioned herein typically are from about 0.01 mg/kg to about 2.0 mg/kg per day, preferably 0.1 mg/kg to 0.7 mg/kg per day, more preferably, 0.5 mg/kg/day. Analogous amounts of other Type 4 or Type 3 phosphodiesterase inhibitors can be determined routinely based on the information given herein, e.g., using the EAE model. However, any amount which is effective in treating Th1-mediated autoimmune disease can be administered to ameliorate or treat the disease. Dosages are determined conventionally, see, e.g., Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Company (1990). The composition may be administered in a

single dose unit or in multiple dosages administered, e.g., twice, three, or four times a day, or by an osmotic pump, which delivers the drug(s) continuously.

Normal dosage amounts may vary from approximately 1 to 100,000 micrograms, up to a total dose of about 10 grams, depending upon the route of administration. Desirable dosages include 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 300, 400, 500, 600, 700, 800, 900, 1000 microgram/kg/day.

Preferable concentrations for these embodiments range from 1 nM to 100,000 nM. For example, preferred concentrations include 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10,000 nM.

Suitably, the weight ratio in the combination of a PDE4 inhibitor to a PDE3 inhibitor varies, without limitation, ordinarily within the range 1:1,000 to 1,000:1, preferably 1:1 to 1,000:1.

Administration

The PDE4 inhibitor (component (i)), and PDE3 inhibitor (component (ii)) combination treatment of the invention can be administered by any conventional means available for the use in conjunction with pharmaceuticals, either as individual separate dosage units administered simultaneously or concurrently, or in a physical combination of each component therapeutic agent in a single or combined dosage unit. The active agents can be administered alone, but are generally administered with a pharmaceutical carrier selected on the basis of the chosen route of administration and standard pharmaceutical practice.

The pharmaceutical compositions according to the present invention are prepared conventionally, comprising substances which are customarily used in pharmaceuticals, e.g., see Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Company (1990), including excipients, carriers, adjuvants and buffers. The compositions can be administered, e.g., parenterally, enterally, orally, intramuscularly, topically, subcutaneously, intravenously, by aerosol, intrathecally directly into the cerebral spinal fluid of the CNS, or preferably by sustained release using, e.g., an implanted mini-osmotic pump (e.g., the

ALZET pump manufactured by ALZA Corporation, P. O. Box 10950, Palo Alto, CA. 94303), or other routes useful to achieve an effect.

Conventional excipients include pharmaceutically acceptable organic or inorganic carrier substances suitable for parenteral, enteral or topical application which do not deleteriously react with the agents. Suitable pharmaceutically acceptable adjuvants include, but are not limited to, water, salt solutions, alcohols, gum arabic, vegetable oils, polyethylene glycols, gelatine, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid monoglycerides and diglycerides, pentaerythritol fatty acid esters, hydroxy-methylcellulose, polyvinyl pyrrolidone, cyclodextrins, etc. The pharmaceutical preparations can be sterilized and, if desired, mixed with stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, coloring, flavoring and/or aromatic substances, etc., which do not react deleteriously with the active compounds.

For parenteral application, particularly suitable are injectable sterile solutions, preferably oil or aqueous solutions, as well as suspensions, emulsions or implants, including suppositories. Ampoules are convenient unit dosages.

For enteral application, particularly suitable are tablets, dragees, suppositories or capsules having talc and/or a carbohydrate carrier or binder. The carrier may be lactose, corn starch, potato starch or a combination thereof. A syrup or elixir may be used when a sweetened vehicle is employed.

The compositions can also be formulated in an aqueous solution, optionally with the addition of additives customary in galenicals, for example, buffers; electrolytes such as sodium chloride; antioxidants such as ascorbic acid; adjuvants, e.g., methyl cellulose, lactose and mannitol and/or surfactants, e.g., lecithins and Tweens and/or aromatic substances for flavoring, e.g., ethereal oils.

The pharmaceutical compositions of the present invention can also comprise other active agents.

Pharmaceutical kits

Pharmaceutical kits useful for the treatment of MS and other autoimmune diseases, which comprise a therapeutically effective amount of a compound of component (i), and a compound of component (ii), in one or more containers, are also within the ambit of the present invention. Sterilization of the container may be carried out using conventional

sterilization methodology well known to those skilled in the art. Component (i), and component (ii) may be in the same container or in separate containers. The containers of materials may comprise separate containers, or one or more multi-part containers, as desired. Component (i), and component (ii), may be separate, or physically combined into a single dosage form or unit as described above. Such kits may further include, if desired, one or more of various conventional pharmaceutical kit components, such as for example, one or more pharmaceutically acceptable carriers, additional vials for mixing the components, etc., as will be readily apparent to those skilled in the art. Instructions, either as inserts or as labels, indicating quantities of the components to be administered, guidelines for administration, and/or guidelines for mixing the components, may also be included in the kit.

The compositions and kits of the present invention may be employed in the treatment of MS and other Th1-mediated autoimmune diseases.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following preferred specific embodiments are, therefore, to be construed as merely illustrative and not limitative of the remainder of the disclosure in any way whatsoever.

The entire disclosures of all applications, patents, and publications cited herein are hereby incorporated by reference.

20 **Effect of PDE4 and PDE3 inhibitors on antigen-specific TCL proliferation**

We examined the effect of rolipram and cilostamide on the proliferation of 47 antigen-specific TCL. Both drugs inhibited antigen-driven TCL proliferation, but only rolipram and the combination of both drugs expressed statistically significant inhibitory effects in a dose dependent manner (Figure 1). The inhibition by rolipram (8.8-32% of inhibition) was more pronounced than that by cilostamide (6.31-22.61% inhibition). Combinations of both drugs (1:1 ratio and half molar concentration of each drug (0.05 μ M, 0.5 μ M and 5 μ M in the final mixture) proved to be most efficient in inhibiting TCL proliferation (10.34-61.05%), exceeding the additive effect of individual drugs. The observed differences between drugs and their combination were statistically significant ($P < 0.05$, Repeated measures ANOVA). Since the above characteristics of the drug combination indicated synergistic effects, we have quantified the degree of synergism

between rolipram and based on the modified Berenbaum equation (Berenbaum, M.C. 1978 *J Infect Dis* 137:122):

The degree of synergism between drugs A and B is equal to $1/R$, where

$$R = [(IC_{20} \text{ drug A} + B)/(IC_{20} \text{ drug A})] + [(IC_{20} \text{ drug B} + A)/(IC_{20} \text{ drug B})]$$

5 Based on the proliferation data in Figure 1 we have estimated the IC_{20} (the concentration of each drug which leads to 20% inhibition of TCL proliferation; this was substituted for IC_{50} as neither rolipram nor cilostamide reached 50% of inhibition) from the dose-response curves and calculated $R_{\text{rolipram} + \text{cilostamide}} = 0.1123$. The degree of synergism between rolipram and cilostamide is $1/R = 8.9$. (Values <1 indicate antagonism, 1 indicates additivity and >1 indicate synergism).

10 **Comparison of the susceptibility to PDE4 and -3 inhibition by autoreactive and foreign-antigen-specific TCL and by TCL derived from MS patients and healthy donors**

15 When the inhibitory effect of PDE inhibitors was assessed after stratification of TCL based on their origin and the type of the selecting antigen (TCL derived from MS patients, 26 TCL, 17 autoreactive, 9 foreign-antigen-specific versus TCL derived from healthy donors, 21 TCL, 5 autoreactive, 16 foreign-antigen-specific) two interesting observations emerged (Figure 2). 1. The MS-derived TCL were more susceptible to the effect of PDE inhibitors than healthy donor-derived TCL ($P < 0.05$ for rolipram 10 μM , cilostamide 10 μM and combination of rolipram + cilostamide 0.1 and 1 μM ; Friedman's repeated measures analysis on ranks). 2. Also there appeared to be a differential sensitivity of autoreactive-TCL and foreign-antigen-specific TCL to the effects of PDE4 and PDE3 inhibition: autoreactive TCL were inhibited by rolipram to a greater extend than foreign-antigen-specific TCL ($P < 0.05$ through the tested concentration range of rolipram, Friedman's repeated measures analysis on ranks). Only stratified data are depicted in 20 Figure 2. Due to the decreased power of a comparison of divided data into individual patient groups, not all differences reached statistical significance ($P < 0.05$, Friedman's repeated measures analysis on ranks) – these are marked with a star.

25 **Effect of PDE inhibitors on cytokine production by antigen-specific TCL**

30 We evaluated the effect of PDE inhibitors on antigen-driven production of two cytokines, IFN- γ (prototypic Th1-) and IL-4 (prototypic Th2-cytokine) (Figure 3). Although a significant variability was noted among individual TCL, the average effect of

PDE4 inhibition by rolipram and PDE4 and PDE3 inhibition by the combination of drugs on IFN- γ production was inhibitory. Rolipram (12.26-27.8% inhibition) and the combination of both drugs (16.07-46.3%) inhibited IFN- γ synthesis in a dose-dependent manner. The effect of cilostamide was mild and did not reach statistical significance for any concentration (4.47-11.27% inhibition). Differences among the drugs were again statistically significant.

Neither rolipram nor the combination of rolipram and cilostamide had statistically significant effects on IL-4 production (inhibition ranging from -0.62 to 1.69 % throughout the concentration range). Cilostamide had a mild inhibitory effect on IL-4 production at 0.1 μ M, whereas high concentrations (10 μ M) significantly enhanced IL-4 production (-11.39% inhibition of 10 μ M concentration). Overall, the effect of PDE4 and PDE3 inhibitors on IFN- γ production paralleled that on proliferation with the drug combination having synergistic effects. IL-4 production was largely unaffected, despite the demonstrated significant inhibition on TCL proliferation at the drug concentrations tested.

15 **Effect of PDE inhibitors on the functional antigen-avidity of TCL**

In order to gain a better understanding of the mechanism of the PDE4 and PDE3 inhibitor-induced bias of immune responses from a Th1 toward a Th2 phenotype, we decided to study their effects on the major components of T cell priming. Apart from the influence on the cytokine milieu, which was supported by the above experiments, we wanted to address the question whether these drugs influence the antigen dose needed for T cell activation, i.e. antigen avidity, and costimulatory signals delivered by antigen-presenting cells (APC). First, to address the question whether the magnitude of the immunomodulatory effect of PDE inhibitors on TCL varies depending on the dose of antigen, we exposed a subgroup of TCL (15 TCL; selected as a representative sample based on their phenotype, the variable susceptibility to PDE inhibition and reactivity to either autoantigen or environmental antigen - Table 1) to 1 μ M concentration of the individual drugs over a wide range of antigen-concentrations. The results of this functional antigen-avidity assay for three TCL (of Th1, Th0 and Th2 phenotypes) are summarized in Figure 4. None of the autoreactive or foreign-antigen-specific TCL that we generated for this project expressed clear Th2 phenotype. We therefore included a Th2-like TCL specific for copolymer-1 (Cop-1), an approved immunomodulatory drug for MS. TCL Th1:MBP and Th0:Flu-HA were representative of all other tested TCL and illustrate the above-mentioned

5 differential effect of PDE inhibition on autoreactive versus foreign-antigen-reactive TCL. All examples also demonstrate the hierarchy of immunomodulation between individual drugs (cilostamide < rolipram < rolipram + cilostamide). For MBP-specific Th1-TCL, a combination of PDE4- and PDE3-inhibition resulted in almost 100% inhibition of proliferation and IFN- γ production. For Flu-HA-specific Th0-TCL, the inhibition of proliferation and IFN- γ production was much less pronounced (around 50% with the combination of drugs for each antigen-concentration) and the production of IL-4 was either unaffected or even enhanced. For Cop-1-specific Th2-TCL, the individual drugs had no effect on antigen-specific proliferation or IL-4 production. Overall, the magnitude of the 10 effect of PDE inhibitors on individual TCL was similar through the tested antigen concentration range. However, as demonstrated in table 1, the PDE inhibition had a mild, but statistically significant effect on the EC₅₀ (concentration of antigen that leads to 50% maximal proliferation) of individual TCL (P=0.009; Friedman's repeated measures analysis 15 on ranks).

15 **Effect of rolipram on the surface expression of costimulatory molecules**

Finally, we wanted to assess the effect of PDE-inhibition on the third important component of T cell priming, the costimulatory signal. We studied the effect of rolipram 20 on the surface expression of costimulatory molecules of human PBMC in the resting and activated state. Freshly isolated PBMC were seeded *ex vivo* with or without rolipram, in a resting state or upon activation with nonspecific stimuli (PHA or LPS). After 12 hours, we 25 analyzed these cells by the three-color flow cytometry. Rolipram consistently downregulated the surface expression of MHC class II both in the resting state and after stimulation with PHA and LPS on monocytes (Figure 5), T- and B-lymphocytes. After 12 hours stimulation of PBMC with LPS, rolipram-treated cultures had decreased surface expression of CD80 (P < 0.001, Mann Whitney Rank Sum test) and increased surface expression of CD86 (P = 0.008; Figure 5). Similar changes in expression of costimulatory molecules were observed on B- and T-lymphocytes, or after stimulation with PHA.

Treatment for Th1-mediated autoimmune disorders

In this disclosure we present a detailed analysis of the effects of the selective PDE4 30 inhibitor rolipram and the PDE3 inhibitor cilostamide on human immune cells to determine the potential of these drugs for the treatment of human Th1-mediated autoimmune disorders. Consistent with the data obtained from animal models (Genain, C.P. *et al.* 1995

PNAS USA 92:3601; Sommer, N. *et al.* 1997 *J Neuroimmunol* 79:54; Ross, S.E. *et al.* 1997 *J Immunol* 159:6253), we demonstrated the predominant inhibitory effect of PDE inhibitors on Th1-mediated immune responses in humans. To address the effect of PDE-inhibitors on the cytokine phenotype of human T cells, we focused our experiments on short-term TCL and performed our analysis on day 22-25 *ex vivo*, in order to avoid possible artifacts of long-term culture. Our data on the modulation of Th2-TCL by PDE-inhibitors are limited, but in agreement with our cytokine data from Th0-TCL (no effect, or even induction of IL-4) and with data from different experimental systems (Lacour, M. *et al.* 1994 *Int Immunol* 6:1333; Xu-Amano, J. *et al.* 1993 *J Exp Med* 178:1309; Munoz, E. *et al.* 1990 *J Exp Med* 172:95); we found no inhibition of antigen-specific TCL proliferation and IL-4 production in these TCL.

Few studies have addressed the question of the effect of PDE-inhibitors on human immune cells. A recent study examined the expression of PDE4 and PDE3 enzymes in autoreactive MBP-specific TCL (Ekholm, D. *et al.* 1997 *J Immunol* 159:1520) and demonstrated that these two families account for the vast majority of PDE enzymatic activity in these cells. This is consistent with our current data, demonstrating a significant inhibition of proliferation at the highest concentration of the drug combination (5 μ M of both rolipram and cilostamide) for virtually all tested TCL. Another study analyzed the influence of rolipram on the functional characteristics of 9 MBP-specific TCL, 5 derived from MS patients and 4 from healthy donors (Pette, M. *et al.* 1999 *J Neuroimmunol* 98:147) and raised the issue of a differential susceptibility of individual TCC to the immunomodulatory influence of rolipram. Although rolipram inhibited TNF- α and - β as well as IL-10 production by TCL, the effect on other cytokines (IFN- γ , IL-4 and IL-13) was inconsistent and did not reach statistical significance. Moreover, Essayan *et al.* suggested a higher susceptibility to PDE4 inhibition by Th2-TCL as compared to Th1-TCL in a limited number of Th1 and Th2 TCL (total of 4) derived from atopic and asthmatic patients (Essayan, D.M. *et al.* 1997 *J Pharmacol Exp Ther* 282:505.). Although it is difficult to compare the data derived from different experimental systems, the reported inhibition of Th1-TCL derived from these asthmatic patients did not reach the magnitude of the typical inhibition of MS-derived Th1 TCL observed in our laboratory using the same concentrations of drugs. Since asthmatic individuals and MS patients have biased immune responses toward opposite T helper phenotypes as compared to unbiased responses of

healthy donors, we asked whether the explanation for these controversial data from human TCL lies in the differential susceptibility to PDE-inhibition between these different patient groups. This hypothesis prompted us to examine the effect of PDE inhibition on large numbers of TCL, derived both from healthy donors and MS patients, and with specificity for two antigens, the autoantigen MBP and the classical foreign recall antigens Flu-HA or tetanus (Gelder, C.M. *et al.* 1996 *J Virol* 70:4787). Indeed, we were able to demonstrate a higher susceptibility to PDE-inhibition by MS-derived- as compared to healthy donor-derived-TCL. This finding has several important implications. It explains how the same therapeutic agent could be considered for the treatment of disorders with potentially different pathogenesis (Th1-mediated autoimmune disorders versus Th-2 mediated asthma and atopic dermatitis). Others recently demonstrated (Sorensen, T.L. *et al.* 1999 *J Clin Invest* 103:807) that the immune system in MS patients is in a "dysregulated state" characterized by an overshooting Th1-response not only to autoantigens, but also to common environmental pathogens. A similar dysregulated state, this time toward Th2-responses, is likely to exist in asthmatic or atopic individuals (Chan, S.C. *et al.* 1993 *J Invest Dermatol* 100:681). If such a dysregulation involves abnormalities in the cAMP second messenger system, it would render TCL derived from these individuals more susceptible to the effects of PDE inhibition, thus at least in part explaining the controversies between results obtained from MS and asthmatic patients. Several reports in the literature indicate that this may be the case. Patients with multiple sclerosis, rheumatoid arthritis or lupus were either found to have low intracellular cAMP levels (Maida, E., and W. Kristoferitsch 1981 *J Neurol* 225:145; Maciejek, Z. *et al.* 1985 *Neurol Neurochir Pol* 19:471), decreased expression and activity of G-protein-coupled receptor kinases (Lombardi, M.S. *et al.* 1999 *Faseb J* 13:715) or deficient type I cAMP-dependent protein kinase A activity (Kammer, G.M. *et al.* 1994 *J Clin Invest* 94:422; Laxminarayana, D. *et al.* 1999 *J Immunol* 162:5639). Similar abnormalities in cAMP signaling were suggested in asthmatic patients (Hanifin, J.M. and S.C. Chan 1995 *J Invest Dermatol* 105:84S; Chan, S.C., and J.M. Hanifin 1993 *J Lab Clin Med* 121:44; Holden, C.A. *et al.* 1986 *J Invest Dermatol* 87:372).

It is more difficult to explain the suggested differential susceptibility to PDE inhibition between autoreactive and foreign-antigen-reactive TCL. We did not find any significant skewing in the cytokine profiles between these two types of TCL that would

account for the observed differential effect. This effect is also not mediated by the need for antigenic processing of MBP as compared to Flu-HA and tetanus peptides, because it was shown that MBP presentation by HLA-DR molecules does not require processing (Vergelli, M. *et al.* 1997 *Eur J Immunol* 27:941), and because we have noted similar effect of PDE-inhibition on 7 MBP-specific TCL stimulated with the peptide epitope. The two likely explanations are: either the signal delivered by the autoantigen may be qualitatively different (partial agonist versus full agonist signal), or the dysregulation in the cAMP system is more pronounced in autoreactive T-cells. We are currently studying this issue in detail. Considering the therapeutic use of PDE inhibitors, the observed higher susceptibility of autoreactive TCL to PDE4-inhibition may widen the therapeutic window in the treatment of autoimmune disorders without inducing general immunosuppression.

In order to explore the possible cause of the PDE-inhibitor-induced bias from Th1 to Th2 phenotype, we decided to study the influence of these drugs on T cell priming. There are three major components contributing to T cell priming, which may influence the phenotype of the primed T cell: the cytokine milieu, the dose and character of the antigen and the costimulatory signal. It was previously demonstrated that selective PDE4 inhibitors or non-selective PDE inhibitors decrease the secretion or expression of proinflammatory cytokines by human mononuclear cells, favoring the cytokine milieu at the time of antigen presentation toward an anti-inflammatory Th2 phenotype (Rieckmann, P. *et al.* 1996 *J Neuroimmunol* 64:193; Weber, F. *et al.* 1998 *Ann Neurol* 44:27; Eigler, A. *et al.* 1998 *J Leukoc Biol* 63:101; Jiang, H. *et al.* 1999 *J Neuroimmunol* 97:134). However, the influences of PDE-inhibition on the other components of T cell priming conditions were unknown. First, we studied the PDE inhibitors in antigen-dose response assays, exploring their effect on the dose of antigen required for T cell activation. The results of these studies indicate that PDE-inhibitors have two different effects on the activation of TCL. They inhibit TCL proliferation irrespective of the antigen dose (Figure 4). On the other hand, the effect of PDE-inhibitors on the EC₅₀ indicates that a higher antigen-dose is necessary for the activation of TCL under their influence. These data suggest that PDE-inhibitors have complex inhibitory effects on T cell activation, most likely by influencing both proximal antigen-responsive events of T cell signaling (Baroja, M. L. *et al.* 1999 *J Immunol* 162:2016) as well as components of more downstream machinery involved in T cell effector functions. The demonstrated influence of PDE4 and -3 on the antigen dose

required for T cell activation, together with the data from the literature that high antigen doses skew the developing immune response toward Th1-, whereas low doses of antigen toward Th2-phenotype (Constant, S.L. and K. Bottomly 1997 *Annu Rev Immunol* 15:297) adds another mechanism for the observed effect of these drugs on Th1/Th2 paradigm.

5 Next, we wanted to assess the influence of rolipram on the third component of T cell priming, the costimulatory signals. It has been suggested that costimulation by CD80 preferentially drives the T cell differentiation toward Th1-responses, whereas CD86-costimulation biases T cell priming toward Th2-responses (Kuchroo, V.K. *et al.* 1995 *Cell* 80:707). Although some concerns were raised regarding the general validity of this 10 dichotomy of the roles of CD80 and CD86 (Lenschow, D.J. *et al.* 1996 *Annu Rev Immunol* 14:233), several reports indicate that the CD80/CD86 costimulatory system is altered in MS patients. Specifically, higher numbers of CD80+ B lymphocytes in the CSF (Svenningsson, A. *et al.* 1997 *J Neuroimmunol* 75:59; Sellebjerg, F. *et al.* 1998 *J Neuroimmunol* 84:179) increased serum levels of CD80+ lymphocytes in patients during MS-exacerbation (Genç, K. *et al.* 1997 *J Clin Invest* 99:2664) and low expression of CD86 on CSF T cells 15 (Sellebjerg, F. *et al.* 1998 *J Neuroimmunol* 84:179) have been reported in patients with MS. Our data indicate that rolipram downregulates CD80 and upregulates CD86 expression on 20 monocytes, B- and T-lymphocytes upon non-specific activation with PHA or LPS. The likely explanation of this observation is the differential kinetic of induction of these costimulatory molecules on APC; CD80 is expressed later than CD86, therefore rolipram may be preventing the switch from CD86 to CD80 expression, a question that merits 25 further study. We also demonstrated that rolipram downregulates MHC class II expression, both in resting conditions and after induction by pro-inflammatory signals. This finding is in agreement with the observation in a murine system where increases in intracellular cAMP inhibit the IFN- γ mediated induction of class II MHC genes (Ivashkiv, L.B. *et al.* 1994 *Immunopharmacology* 27:67). Together these changes in the costimulatory profile on 30 APC favor T cell priming from Th1 toward Th0 or Th2 phenotypes and may limit the effective presentation of autoantigen in inflammatory MS lesions. Indeed, rolipram was shown to reduce the number of IFN- γ secreting cells upon priming of human mononuclear cells in bulk cultures to the autoantigen MBP, while the numbers of IL-4 or IL-10 secreting cells were unaffected (Navikas, V. *et al.* 1998 *Clin Neuropharmacol* 21:236).

The data here demonstrate a favorable drug profile of PDE4- and PDE4 co-inhibitors with PDE3-inhibitors for the treatment of Th1-mediated autoimmune disorders, however the question remains whether this immunomodulatory effect is expressed within a concentration range that is achievable in humans *in vivo*. The concentrations of rolipram achievable in healthy volunteers following 0.75 mg three times daily and 1.5 mg three times daily were 24 ng/ml and 53 ng/ml (0.09 - 0.2 μ M). Our data, summarized in Figure 6, indicate that rolipram and especially the combination of rolipram and cilostamide have mild immunomodulatory effect at the concentration of 0.1 μ M. However, due to the immunomodulation at multiple levels (influence on T cell priming conditions, antigen-specific proliferation and cytokine production) the *in vivo* effect is likely to be more prominent. The combination of PDE4 and PDE3 inhibitors express a high degree of synergism that is, to our experience, achievable even at much lower concentrations of cilostamide in the final mixture. Therefore, the combination of PDE4 inhibitors with relatively small amounts of PDE3 inhibitors is envisioned as representing a way for broadening the therapeutic window in the treatment of human disorders. The molecular mechanism of this synergy is not known. It has been reported that PDE3 inhibitors alone have little effect on the total intracellular cAMP levels and they do not further enhance the cAMP accumulation induced by rolipram (Denis, D. and D. Riendeau 1999 *Eur J Pharmacol* 367:343). However, it has been suggested, that PDE3 (predominantly localized to the particulate cellular fraction) and PDE4 (predominantly cytosolic) may regulate different pools of cAMP (Chini, C.C. *et al.* 1997 *J Biol Chem* 272:9854; Verghese, M.W. *et al.* 1995 *Mol Pharmacol* 47:1164). It is conceivable that intracellular signaling can partially adapt to the effects of PDE4 inhibition by diverting critical pathways blocked by high cytosolic cAMP and activation of PKA to the alternative pathways, which in turn may be affected by PDE3 inhibition. Elucidating the molecular mechanism of this synergy between PDE4 and PDE3 inhibition will enhance our understanding of cAMP second messenger signaling.

EXAMPLE 1

Reagents, generation of T cell lines, proliferation assays

Rolipram (racemate of 4-(3'-cyclopentyloxy-4'-methoxyphenyl)-2-pyrrolidinone) was kindly provided by Dr. Harald Dinter (Berlex Laboratories, Richmond, CA), cilostamide (OPC 3689) was a kind gift of Dr. Vincent Manganiello (PCCMB, NHLBI,

NIH, Bethesda, MD). Fresh solutions of individual drugs were prepared for each experiment. DMSO (Sigma, St. Louis, MO), the solvent for both drugs was used in 1:1000 dilution with T cell media for 10 μ M concentration of drugs and at this concentration did not influence T cell proliferation when used as a negative control.

5 Myelin basic protein was prepared as described (Deibler, G.E. *et al.* 1972 *Prep Biochem* 2:139). Peptides Flu-HA (306-318), Tetanus (830-843) were synthesized by continuous flow, solid phase peptide synthesis on the basis of the F-moc/Bu' strategy. Peptides were purified by HPLC and their identities were tested using ion spray mass spectrometry.

10 TCL were generated by IL-7-modified primary proliferation assay, a method which allows the rapid expansion of antigen-specific T cells, including *in vivo* activated cells. Briefly, peripheral blood mononuclear cells (PBMC) were isolated from fresh leukaphereses by Ficoll density gradients and were seeded in 96-well U-bottom plates in T cell medium (IMDM (Gibco, Grand Islands, NY) containing 2 mM L-glutamine, 50 μ g/ml 15 gentamicin and 100 U/ml penicillin/streptomycin (all Whittaker Bioproducts, Gaithesburg, MD) and 5% pooled human plasma) at 1 \times 10⁵ cells/well with addition of IL-7 (recombinant human IL-7; Pepro-Tech Inc., Rocky Hill, NJ) 10 ng/ml. After 7 days (37°C and 5% CO₂), cultures were split by transferring 100 μ l of each cell culture into a "daughter plate", which was pulsed with ³H-thymidine (Amersham, Arlington Heights, IL) at 1 μ Ci/well. The incorporated radioactivity (counts per minute, CPM) was measured by 20 scintillation counting (Betaplate, Pharmacia LKB, Piscataway, NJ) 8 hours later. Proliferation of cultures with antigen (MBP 25 μ g/ml or peptides 5 μ g/ml) was compared with the proliferation of negative control wells seeded without antigen. Positive cultures (stimulation index (SI) greater than 2 and absolute CPM at least 3 standard deviations 25 above the average CPM of negative control wells) were identified on the "mother plates" and were periodically restimulated. Antigen-specificity was confirmed at the end of the 2nd *in vitro* stimulation cycle in 48-hour proliferation assays as described (Muraro, P.A. *et al.* 1997 *J Clin Invest* 100:339). All blood samples were collected according to an IRB-approved protocol, and informed consent was obtained prior to study. None of the patients 30 received any immunomodulatory or immunosuppressive treatment within 1 month prior to blood collection.

EXAMPLE 2**Effect of PDE4 and PDE3 inhibitors on antigen-specific TCL proliferation and functional antigen-avidity**

The effect of PDE inhibitors on antigen-specific proliferation, cytokine production and functional antigen-avidity was assessed during the 3rd stimulation cycle (days 22-25 *ex vivo*). Each drug was used alone (in three concentrations; 0.1 μ M, 1 μ M and 10 μ M) or in combination (0.05 μ M, 0.5 μ M and 5 μ M concentration of individual drugs in the mixture). The selected dose range included the concentrations achievable *in vivo* (for rolipram 0.09 and 0.2 μ M). Each condition was tested in duplicate, including negative- (no antigen) and positive control (antigen, no drug). T cells were plated in 96 well U-bottom plates at 2 x 10⁴ T cells/well with irradiated autologous PBMC at 1 x 10⁵ cells/well. Antigen was added at the seeding concentration (MBP 25 μ g/ml, peptides 5 μ g/ml) or over a wide range of antigen concentrations for the functional antigen avidity assay. Supernatants from these assays were collected after 36 hours of incubation and stored frozen till analysis. For the last 8 hours of incubation, cells were pulsed with ³H-thymidine at 1 μ Ci/well, and the incorporated radioactivity was measured by scintillation counting.

EXAMPLE 3**Cytokine secretion**

Secretion of a Th1 (IFN- γ) and Th2 (IL-4) cytokine by antigen-specific TCL was assessed by sandwich ELISA (Cyto-Sets from BioSource International, Camarillo, CA) according to manufacturer's recommendation. All standards and samples were tested in duplicates.

EXAMPLE 4**Flow cytometry (FACS) analysis of the surface expression of costimulatory molecules**

Fresh PBMC (1.2 x 10⁶ cells/ml) were seeded in bulk cultures in 48 well plate, with or without rolipram 10 μ M. In addition to non-stimulated cells, the effect of rolipram was assessed upon non-specific stimulation with lipopolysaccharide (LPS; 2.5 μ g/ml) or phytohemagglutinin-P (PHA; 5 μ g/ml, both from Sigma, St. Louis, MO). After 12 hours incubation cells were washed with wash buffer (Dulbecco's PBS with 1% heat inactivated FCS and 0.1% sodium azide) and incubated with fluorescein-, phycoerythrin- or Cy-Chrome-conjugated antibody (HLA-DR,DP,DQ-FITC, CD19-FITC, CD14-FITC and -PE,

CD80-FITC and -PE, CD86-FITC and -PE, CD3-Cy-Chrome - all from PharMingen, San Diego, CA) at saturating concentrations for 30 minutes on ice, then washed 3 times and analyzed (FACScan, Beckton-Dickinson, CA) using Cell-Quest software. Isotype-matched mouse IgG negative controls were used for each staining. Monocytes were gated based on the size-characteristics (forward and side-scatter), and expression of CD14. Lymphocytes were identified by the size-characteristics and differentiation between T and B-lymphocytes was based on the expression of CD3 and CD19 molecules respectively. 5000 cells in gated population were analyzed per sample.

EXAMPLE 5

10 Statistical analysis

The data were analyzed by a commercial software package (Sigma-Stat, SPSS Inc, Chicago, IL). The effect of the drugs on biological functions of TCL was evaluated by one way repeated measures ANOVA or, if normality failed by Friedman's repeated measure analysis on Ranks. Statistically significant differences from repeated measures ANOVA 15 were further analyzed by Student-Newman-Keuls test with $P<0.05$ as a cut-off for statistical significance. The effect of rolipram on costimulatory molecules was assessed by Mann Whitney Rank Sum test.

20 From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention and, without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

WHAT IS CLAIMED IS:

1. A pharmaceutical composition comprising a combination of a PDE4 inhibitor and a PDE3 inhibitor in a synergistic combination and a pharmaceutically acceptable vehicle.

5 2. A kit comprising a combination of a PDE4 inhibitor and a PDE3 inhibitor in a synergistic combination.

3. A method of modulating Th1-mediated immunity comprising administering to a host in need thereof a composition comprising an effective amount of a combination of a PDE4 inhibitor and a PDE3 inhibitor in a synergistic combination, whereby Th1-mediated 10 immunity is modulated.

4. The composition, kit or method of any of Claims 1-3 wherein said PDE4 inhibitor is a member selected from the group consisting of rolipram, RO 20-1724 (4-[(3-butyoxy-4-methoxyphenyl)methyl]-2-imidazolidinone), ICI 63197 (2-amino-6-methyl-4-propyl[1,2,4]triazolo[1,5-a]pyrimidin-5(4H)-one), denbufylline, EMD 54622, 15 denbufylline, etazolate, Org 30029, and zardaverine.

5. The composition, kit or method of Claim 4 wherein said PDE4 inhibitor is rolipram.

6. The composition, kit or method of any of Claims 1-3 wherein said PDE3 inhibitor is a member selected from the group consisting of cilostamide, milrinone, 20 piroximone, pimobendan, imazodan, zardaverine, enoximone, indolidan, motapizone, SK&F94120, RS82856, ORg 30029, ICI 118233, and EMD 54622.

7. The composition, kit or method Claim 6 wherein said PDE3 inhibitor is cilostamide.

8. The method of Claim 3 wherein modulation is measured by cytokine 25 production.

9. The method of Claim 3 wherein modulation is measured by IFN- γ production.

10. The method of Claim 3 wherein modulation is measured by macrophage proliferation.

30 11. The method of Claim 3 wherein a combination of 0.01 mg/kg - 2.0 mg/kg per day of PDE4 inhibitor is administered in combination with 0.01 mg/kg - 2.0 mg/kg per day of PDE3 inhibitor.

12. The method of Claim 3 wherein a combination of 0.1 mg/kg - 0.7 mg/kg per day of PDE4 inhibitor is administered in combination with 0.1 mg/kg - 0.7 mg/kg of PDE3 inhibitor.

5 13. The method of Claim 3 wherein a combination of a PDE4 inhibitor is administered in combination with a PDE3 inhibitor in a weight ratio of 1:1 to 1,000:1.

14. The method of Claim 3 wherein the effective amount is administered as a single dose.

10 15. The method of Claim 3 wherein the effective amount is administered in multiple doses.

16. The method of Claim 3 wherein said composition is administered to a human in need thereof.

17. The method of Claim 3 wherein said composition is administered intramuscularly, subcutaneously, intravenously, or intrathecally.

15 18. The method of Claim 3 wherein said composition is administered by sustained release.

19. The method of Claim 3 wherein said composition is administered by implanted osmotic pump.

20 20. A method of ameliorating a Th1-mediated autoimmune disease comprising administering to a host in need thereof a composition comprising an effective amount of a combination of a PDE4 inhibitor and a PDE3 inhibitor in a synergistic combination, whereby said Th1-mediated autoimmune disease is ameliorated.

21. The method of claim 20 wherein said disease is multiple sclerosis.

22. The method of claim 20 wherein said disease is neuromyelitis optica.

23. The method of claim 20 wherein said disease is diffuse sclerosis.

25 24. The method of claim 20 wherein said disease is transitional sclerosis.

25 25. The method of claim 20 wherein said disease is acute disseminated encephalomyelitis.

26. The method of claim 20 wherein said disease is optic neuritis.

27. The method of claim 20 wherein said disease is diabetes mellitus.

30 28. The method of claim 20 wherein said disease is rheumatoid arthritis.

29. The method of claim 20 wherein said disease is uveitis.

30. A method of ameliorating asthma comprising administering to a host in need thereof a composition comprising an effective amount of a combination of a PDE4 inhibitor and a PDE3 inhibitor in a synergistic combination, whereby said asthma is ameliorated.

Figure A

Selection of Th1 or Th2 Mediated Immunity

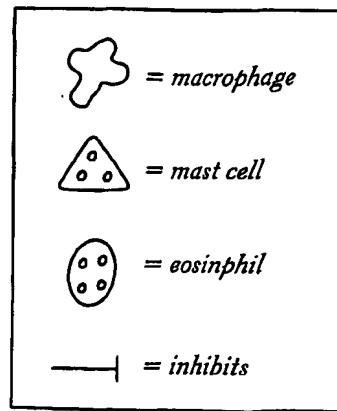
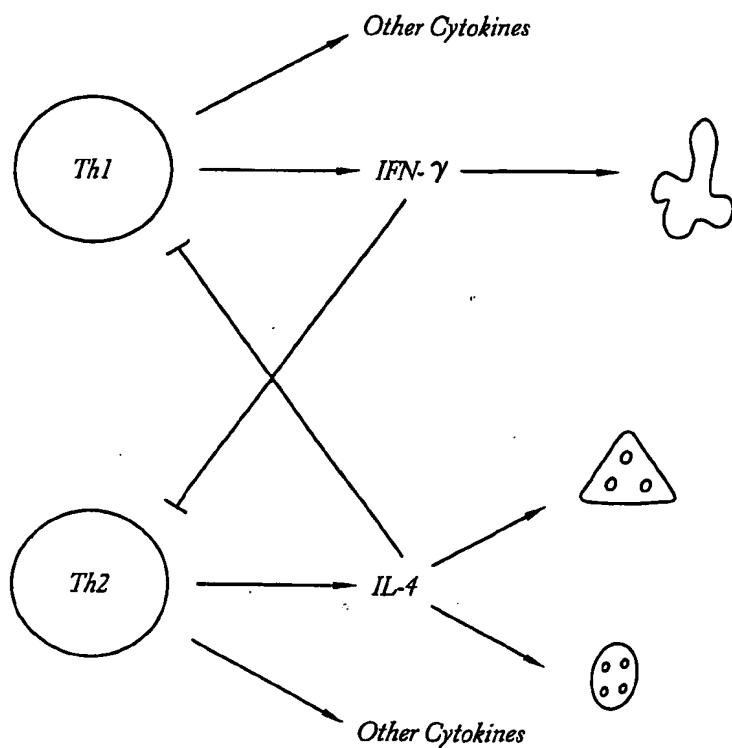


Figure B

Effect of PDE4 inhibitors (rolipram) on Th1 and Th2 Mediated Immunity

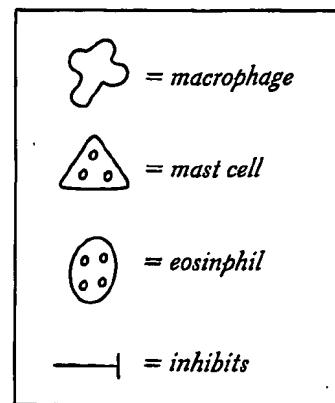
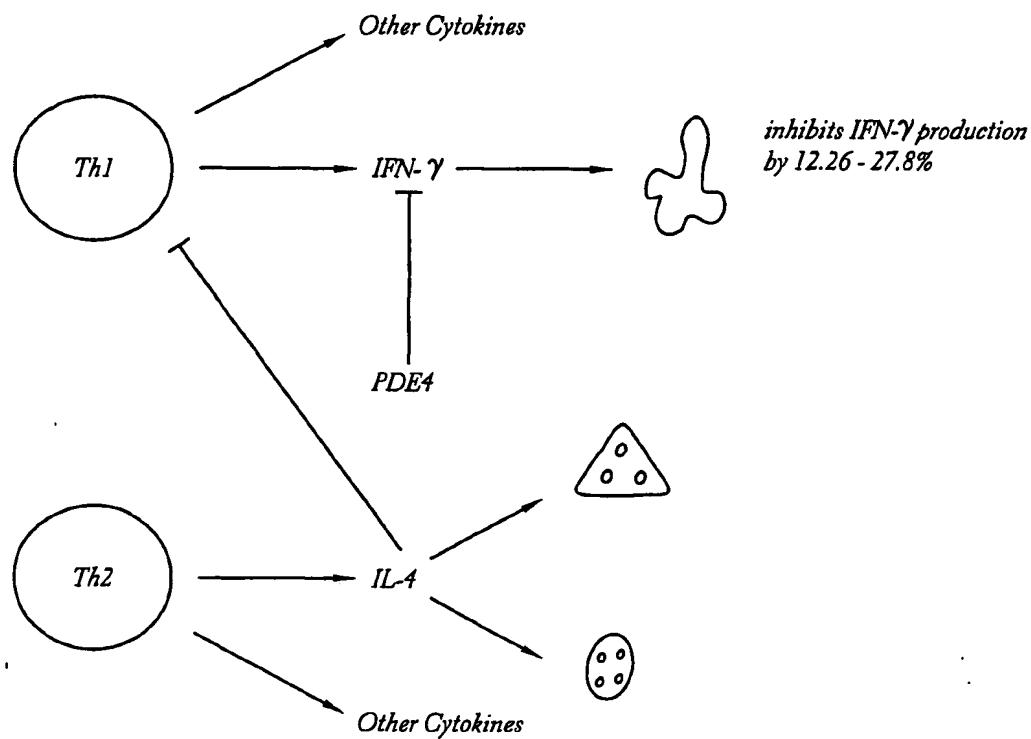


Figure C

Effect of PDE3 (cilostamide) on Th1 and Th2 Mediated Immunity

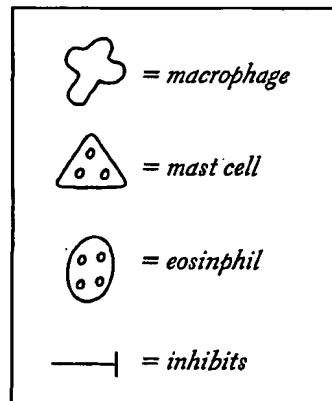
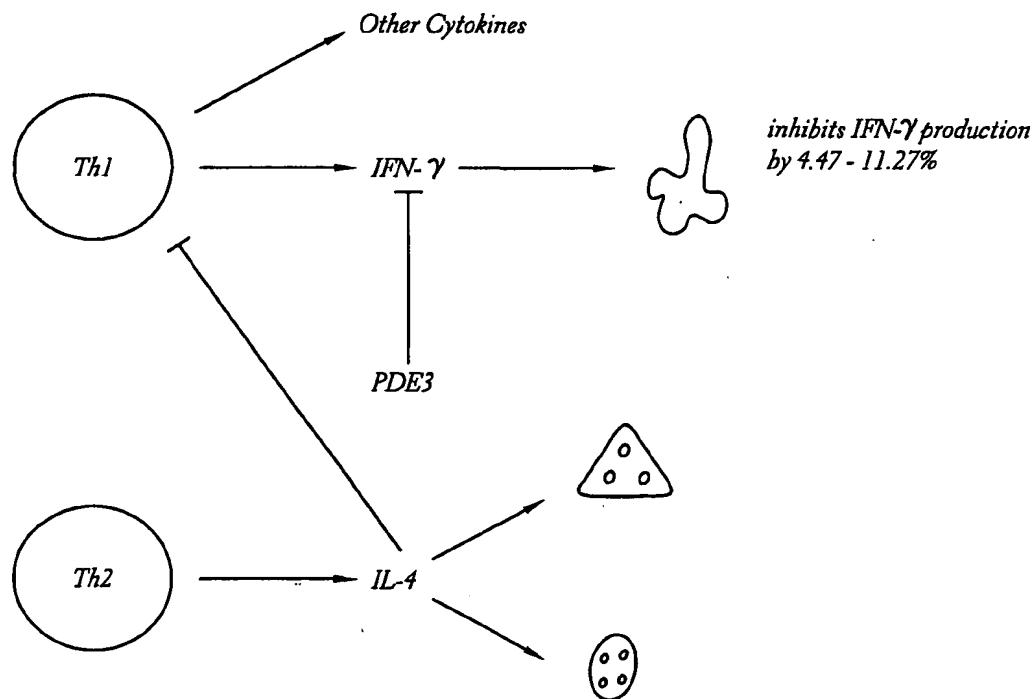
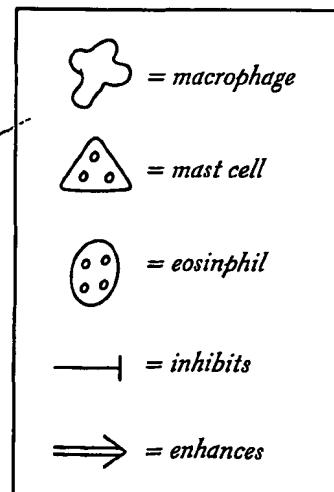
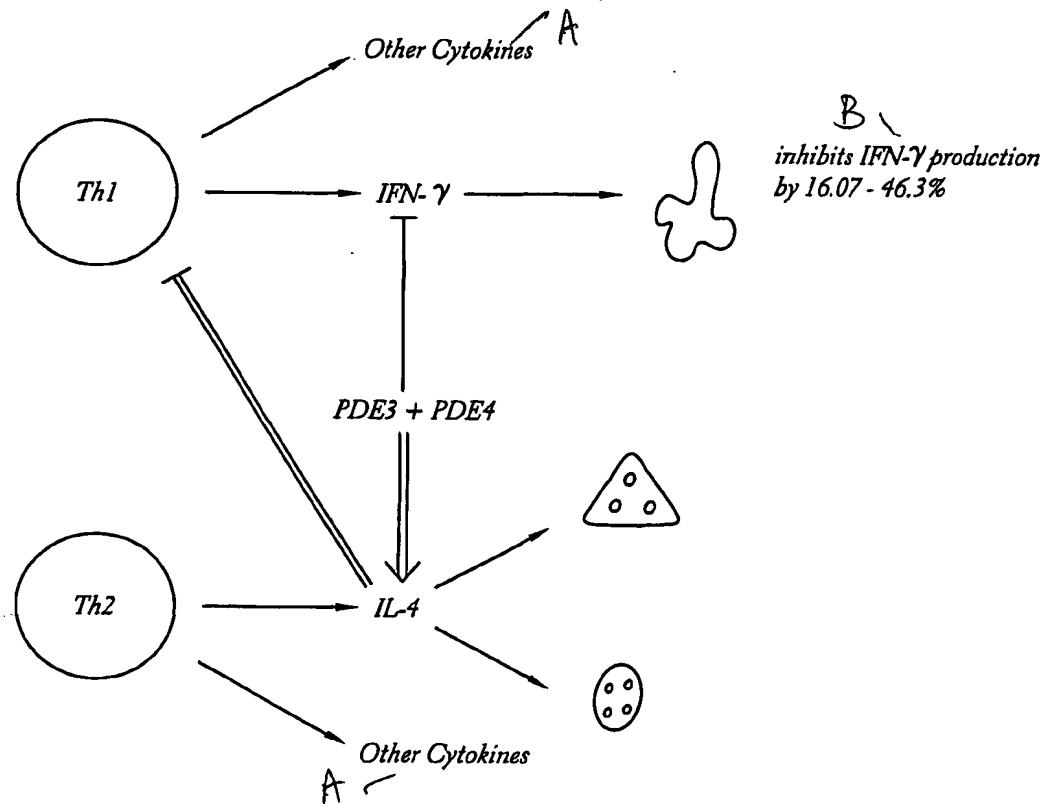


Figure D

Effect of PDE3 (cilostamide) and PDE4 (rolipram) on Th1 and Th2 Mediated Immunity



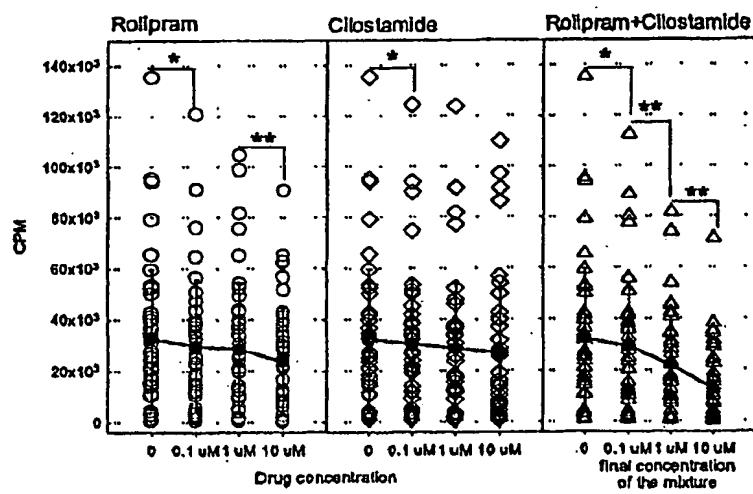


Figure 1

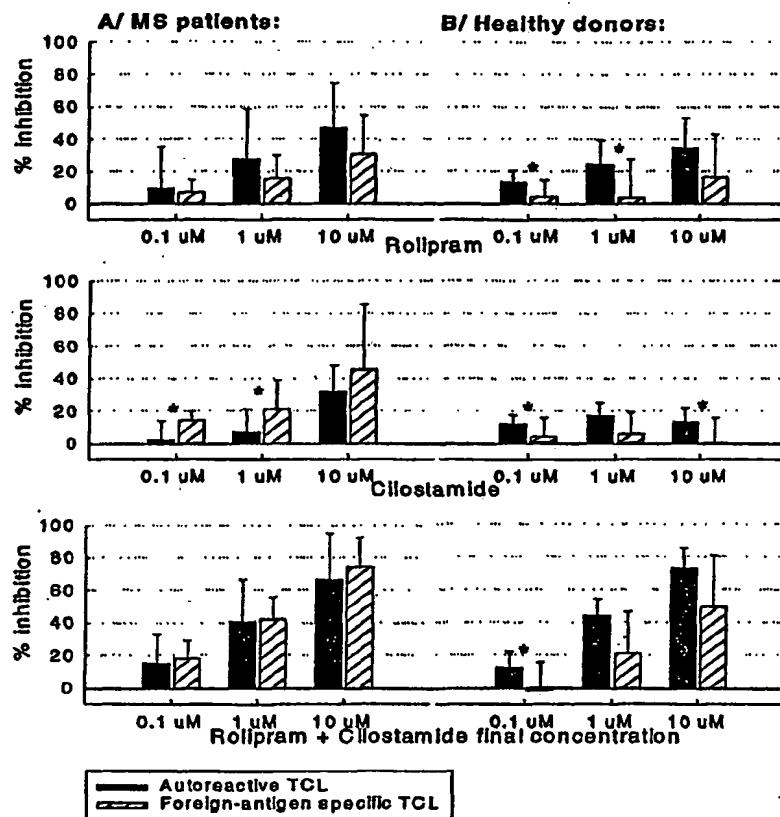


Figure 2

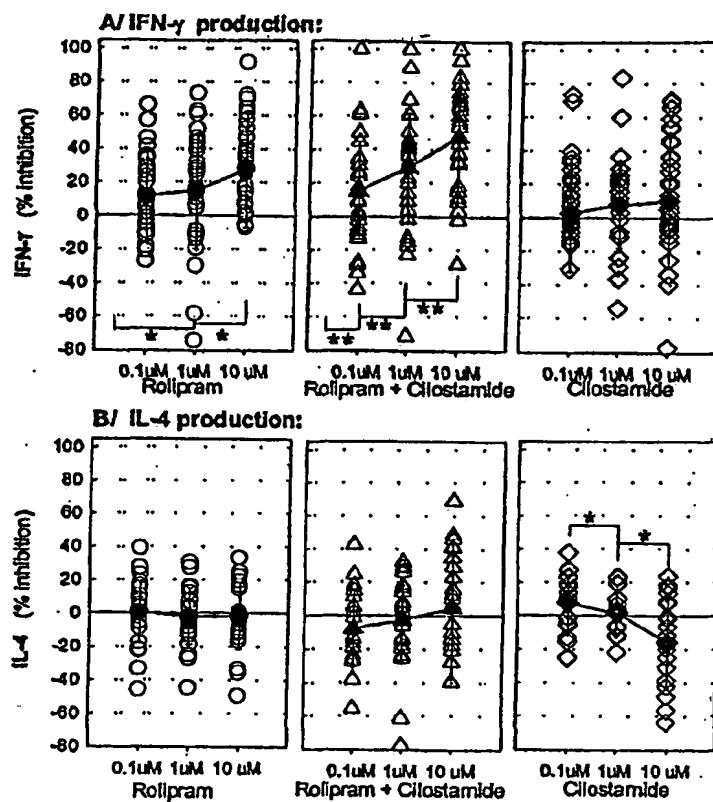


Figure 3

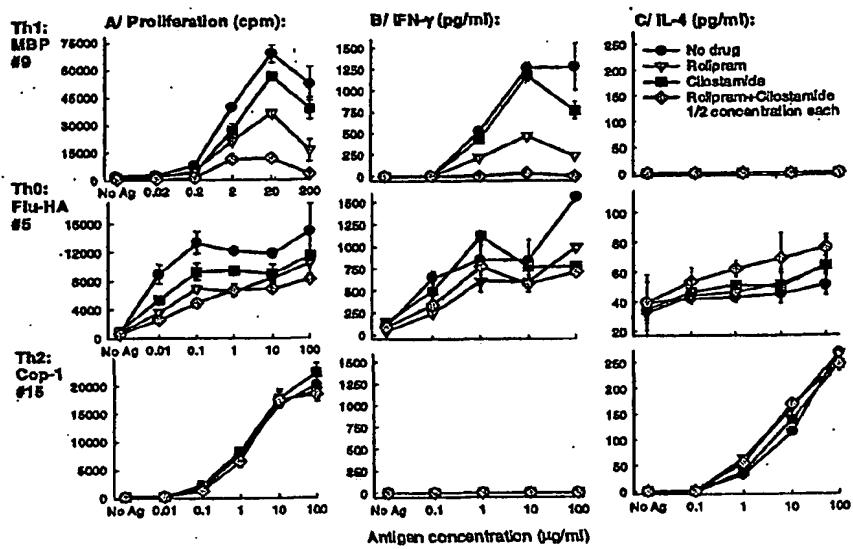


Figure 4

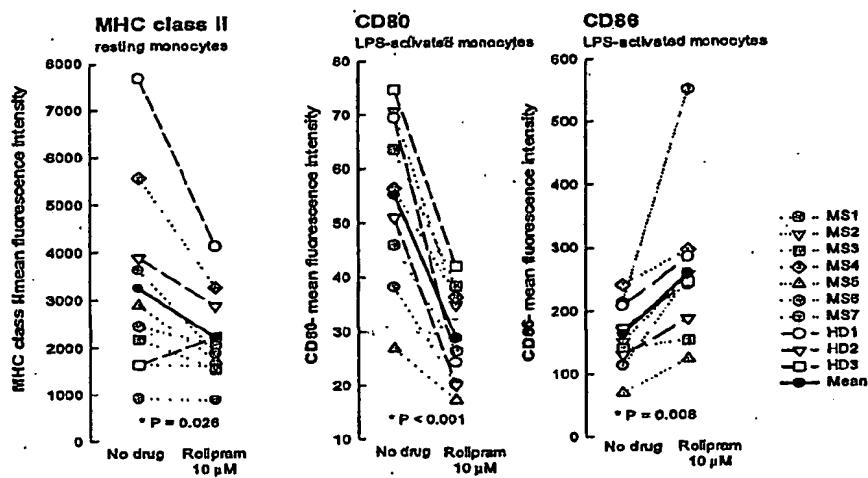


Figure 5

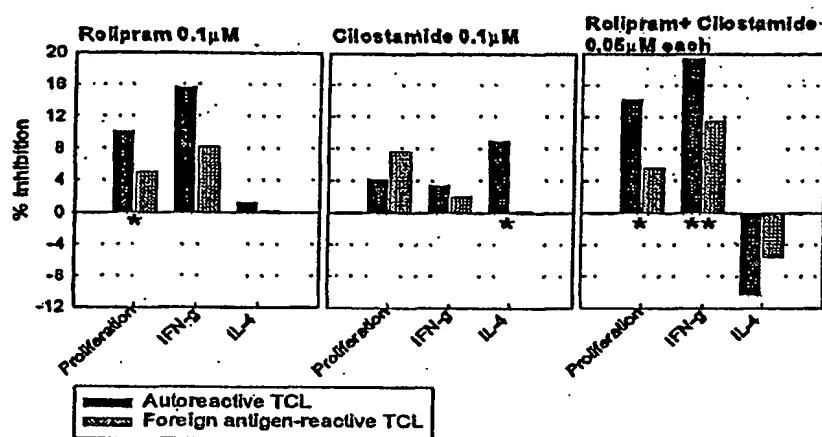


Figure 6

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 01/49693

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 7 A61P37/02 A61K31/4015 A61K31/435

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHEDMinimum documentation searched (classification system followed by classification symbols)
 IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EMBASE, EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, PASCAL, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00 66123 A (BYK GULDEN LOMBERG CHEM FAB ;KILIAN ULRICH (DE)) 9 November 2000 (2000-11-09) page 2, paragraphs 1,5 page 3, paragraphs 3,6 page 4, paragraph 1 – paragraph 3 —	1,2,6
X	BIELEKOVA B. ET AL: "Therapeutic potential of phosphodiesterase-4 and -3 inhibitors in Th1- mediated autoimmune diseases." JOURNAL OF IMMUNOLOGY, (15 JAN 2000) 164/2 (1117-1124). XP002198064 the whole document —	1,2,4-7

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Date of the actual completion of the international search

6 May 2002

Date of mailing of the International search report

04/06/2002

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PAN X ET AL: "SYNERGISTIC INTERACTIONS BETWEEN SELECTIVE PHARMACOLOGICAL INHIBITORS OF PHOSPHODIESTERASE ISOZYME FAMILIES PDE III AND PDE IV TO ATTENUATE PROLIFERATION OF RAT VASCULAR SMOOTH MUSCLE CELLS" BIOCHEMICAL PHARMACOLOGY, PERGAMON, OXFORD, GB, vol. 48, no. 4, 17 August 1994 (1994-08-17), pages 827-835, XP000999727 ISSN: 0006-2952 abstract; figure 7 -----	1, 2, 4
P, X	WO 01 35979 A (ICOS CORP ;SNYDER PETER (US)) 25 May 2001 (2001-05-25) page 4 example 1 -----	1, 2, 4-7

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. Application No

PCT/US 01/49693

Patent document cited in search report	Publication date		Patent family member(s)		Publication date
WO 0066123	A 09-11-2000	AU WO EP	4558800 A 0066123 A1 1176960 A1		17-11-2000 09-11-2000 06-02-2002
WO 0135979	A 25-05-2001	AU WO	3270301 A 0135979 A2		30-05-2001 25-05-2001

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